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ORIGINAL ARTICLE

Role of oxygen free radicals in the proliferation of myofibroblasts induced by AngII

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Abstract Previous studies have demonstrated the important role of angiotension II (AngII) in promoting proliferation of myofibroblasts (myoFbs) and myocardial fibrosis. However, the underlying mechanisms and the role of oxygen free radicals in the proliferation of myofibroblasts induced by AngII are unclear. The present study was designed to shed light on this issue through exploration of AngII signaling pathways *via in vitro* experiments. Primary cultures of neonatal rat myoFbs were divided into five groups which were treated with AngII (10^{-8} to 10^{-6} M), AngII with the antioxidant *N*-acetyl-*L*-cysteine (NAC), or normal culture medium. We observed the proliferation of myoFbs as induced by AngII at different concentrations with MTT. Reactive oxygen species (ROS) levels in myoFbs were detected by monitoring the fluorescence of 2′,7′-dichlorofluorescein. The contents and levels of oxygen free radicals ($OH^{\bullet}$) in the three groups were detected by spectrophotometer, immunocytochemical staining, and confocal fluorescence. Western blot and image analysis were used to measure membrane translocation and expression of phospho-protein kinase Cα. MyoFbs incubated with AngII (10^{-8} to 10^{-6} M) for 24 h increased their rate of proliferation, the content of $OH^{\bullet}$, and expression of ROS ($P<0.01$ *vs.* control group), whereas these parameters decreased in the presence of NAC. Immunocytochemistry, confocal fluorescence staining and image analysis showed that AngII could promote the translocation and expression of *p*-PKCα in membrane, and the antioxidant NAC blocked this increase ($P<0.01$). Western blot results also showed that NAC could inhibit the expression of *p*-PKCα.

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1. Introduction

Myocardial fibrosis is seen in several pathological conditions, such as hypertension, myocardial infarction and heart failure[1]. In a normal heart, myocardial cells and myofibroblasts (myoFbs) are the major components of heart tissue. In disease conditions abnormal proliferation of myoFbs can increase the accumulation of interstitial collagens and lead to myocardial fibrosis, resulting in myocardial stiffness that ultimately disrupts the coordination of myocardial excitation-contraction coupling in both diastole and systole[1–5].

Protein kinase C (PKC) is a group of closely related serine–threonine protein kinases expressed in all tissues. Members of the PKC family are important signal transducers in virtually every mammalian cell type. In the heart, PKC isozymes were thought to participate in a signal network that programs developmental and pathological myoFbs proliferation. The mRNA expression of PKCs is found in cultured rat cardiomyocytes. PKC isozymes are involved in a variety of chronic cardiac diseases as well as in acute cardiac injuries and preconditioning cytoprotection. Interference with such signal pathways may result in inhibition of myoFbs proliferation, myocardial fibrosis and reverse cardiac remodeling.

Much research has shown that the stress produced by oxygen radicals on cardiovascular cell toxicity is an important component of many cardiovascular diseases[6,7]. However, the recent literatures reported that free radicals generated during oxidative stress may act as intracellular signal transduction molecules and are involved in the regulation of cell function[8,9]. In this study, oxidative stress of myoFbs was induced by angiotensin II (AngII), and the ability of *N*-acetylcysteine (NAC) to prevent this proliferation was determined. Our results demonstrate that NAC can inhibit the proliferation of myoFbs induced by AngII, perhaps by decreasing the content of oxygen free radicals ($OH^{\bullet}$) and inhibition of the membrane translocation and expression of *p*-PKCα. Identification of the signaling pathways involved in myocardial remodeling in response to AngII may reveal new therapeutic targets in the prevention and treatment of cardiovascular diseases.

2. Materials and methods

2.1. Materials

Wistar rats of 1–3 days old were purchased from the Center of Experimental Animals of Jilin University (Certificate No. of animals was SCXK (JI) 2007-0003). AngII and anti-β-actin were from Sigma Chemicals (St. Louis, USA). PKCα antibody (anti-PKCα goat polyclonal antibody) was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat secondary antibodies with fluorescein isothiocyanate (FITC) were from BOSTER Bio-Technology Co., Ltd. (Wuhan, China). Iscove's modified Dulbecco's medium (IMDM) was from GIBCO. Fetal bovine serum (FBS) and immunohistochemistry kit were from YHJM (Beijing, China), and trypsin was from Biotech (Changchun, China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemicals (St. Louis, USA). A reactive oxygen species assay kit was obtained from Biotech (Biyuntian, China), and $OH^{\bullet}$ was purchased from Biological Engineering Company (Shanghai, China). All chemicals used in the present study were of analytical grade.

2.2. Cell culture

MyoFbs of 1–3 days old Wistar rats were obtained by digestion with 0.05% trypsin of cardiac tissues at 37 °C for 5 min. The supernatants were collected and centrifuged at 1200 rpm for 10 min, with supernatant discarded. The cell pellet was resuspended in IMDM containing 10% FBS and plated in a culture flask and incubated for at least 90 min at 37 °C in a 5% CO_2 incubator. MyoFbs adhered to the culture flask surface while the cardiocytes remained unattached and were discarded in the supernatant. MyoFbs with IMDM containing 10% FBS were cultured for 48 h at 37 °C in a 5% CO_2 incubator, and then myoFbs were digested with 0.25% trypsin. When fibroblasts were covering the culture flask surface, the cells were diluted to 1.0×10^5 cells/L and seeded in 24 and 96-well plates for immunocytochemistry and MTT assay, or diluted to 1.0×10^8 cells/L and plated in 100-mL glass flasks for Western blot assay. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed after 48 h.

2.3. Treatment protocols

MyoFbs were cultured for 48 h, and then these cells were subjected to drug treatment for 24 or 48 h. Cells were serum-starved for 12 h before drug treatment (myoFbs will tend to synchronization before they are treated, due to myoFbs having higher ability to divide). MyoFbs were assigned to 5 groups as follows. Group A (control): cardiac fibroblasts were cultured in 1% FBS for 24 h/48 h; group B (AngII: 10^{-6} M): cardiac fibroblasts were subjected to AngII (10^{-6} M) and 1% FBS for 24 h/48 h; group C (AngII: 10^{-7} M): cardiac fibroblasts were subjected to AngII (10^{-7} M) and 1% FBS for 24 h/48 h; group D (AngII: 10^{-8} M): cardiac fibroblasts were subjected to AngII (10^{-8} M) and 1% FBS for 24 h/48 h; group E (NAC, AngII): cardiac fibroblasts were subjected to a mixture of NAC (10^{-4} M), AngII (10^{-7} M) and 1% FBS for 24 h/48 h.

2.4. Cell growth and proliferation assay

The growth and proliferation of myoFbs was determined by the MTT assay. Cells (1×10^5 cells/mL) were seeded into 96-well plates and cultured in IMDM with 10% FBS for 36 h. They were exposed to various treatments for 12, 24 and 36 h. After treatment, 10 μL of MTT solution (5 mg/mL in PBS) was added in each well, and the cells were incubated for another 4 h at 37 °C. The medium then was discarded and 150 μL dimethyl sulfoxide (DMSO) was added to each well for the dissolution of formazan crystals. The absorbance of each well was read at 490 nm with a Microplate Reader. The cell proliferation rate was expressed as a percentage of the control rate.

2.5. The contents of $OH^{\bullet}$

MyoFbs were washed three times with cold PBS after treatment with NAC and AngII for 24 h, and then were diluted with PBS to a cell concentration of about 10^8 cells/mL. By repeated freezing and thawing so that myoFbs were lysed and released intracellular $OH^{\bullet}$. MyoFbs were centrifuged at 2000–3000 rpm for 20 min and the supernatant was collected to measure the content of $OH^{\bullet}$ by a hydroxyl radical ELISA kit in accordance with instructions for operation.

2.6. *Immunohistochemistry and immunofluorescein staining*

MyoFbs were treated with NAC and AngII for 24 h, washed three times with PBS at 37 °C for 5 min, fixed with 4% paraformaldehyde for 30 min, and then washed three times with distilled water for 5 min each. Cells were lysed with 0.3% Triton-X 100 for 15 min. MyoFbs were incubated with 1% bovine serum albumin (BSA) for 30 min and then washed three times with distilled water for 5 min each. A mixture of anti-phosphate protein kinase C at 1:100 was added at 300 μL per pore to incubate at 4 °C overnight. After three washes with PBS for 5 min each, the goat anti-rabbit secondary antibodies labeled with FITC were diluted with PBS at 1:50 and added to the plates and incubated at 37 °C for 30 min. The cells were then further incubated with 300 μL of propidium iodide (PI) before being washed with PBS for 5 min and were immediately analyzed under a laser confocal scanning microscope. The membrane of myoFb stained green was measured *via* the Image-pro plus V5.0. The mean density was measured from 6 cells and determined by computer morphometry with the Image-pro plus software.

2.7. *Western blot analysis*

MyoFbs were washed three times with cold PBS after treatment for 48 h and then lysed at 4 °C in ice-cold immunoprecipitation lysis buffer containing All-in-One (phosphatase inhibitor mixtures, Beyotime Biotechnology) and PMSF (final concentration at 10 mM) for 30 min. Cell lysates were centrifuged at 12,000 × *g* for 10 min and protein concentrations in the supernatants were quantified with Coomassie brilliant blue. Protein samples (20 μg) were separated by 12% denaturing SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by electrophoretic transfer. The membranes were blocked in 5% BSA in PBS for 2 h, washed three times with 0.05% Tween-20 (PBS-T) for 10 min each, and then incubated with primary antibody (anti-*p*-PKCα antibodies at a dilution of 1:200 in PBS, anti-tubulin at 1:5000 dilution) at 4 °C overnight. Having been washed three times with PBS-Tween-20 for 10 min each, the membranes were incubated for 2 h in diluted secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibodies, 1:1000 dilution) for 2 h. After washing three times with PBS-T for 10 min each, the bands were detected by DAB, then analyzed on a Tanon GIS gel image processing system.

2.8. *Statistical analysis*

Data were expressed as mean ± SD. The mean values were compared using Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. *Promotion by AngII and inhibition by NAC of the growth and proliferation of myoFbs*

To investigate whether the effect of AngII on proliferation is related to time and concentration, different concentrations and times of exposure of AngII were examined. In Table 1, after treatment with AngII (10^{-8} to 10^{-6} M) and NAC (10^{-4} M) for different times (12, 24 and 36 h), proliferation of myoFbs was measured by MTT. In the AngII (10^{-7} M and 24 h) group, cell proliferation of myoFbs was 0.483 ± 0.12. Concentration (10^{-7} M) and time (24 h) of AngII affected the proliferation of myoFbs, compared with control group ($P<0.01$). MyoFbs proliferation was not evident at AngII (10^{-8} and 10^{-6} M) for 12 h or 36 h, compared with control group; the NAC (10^{-4} M) group proliferation rate was 0.357 ± 0.13 (24 h). The above data show that the proliferative effect of AngII depended on concentration and time.

3.2. *Effect of NAC on $OH^{\bullet}$ content of myoFbs*

After the treatment of myoFbs with NAC and AngII for 24 h, the content of $OH^{\bullet}$ of myoFb was significantly increased in AngII group (93.53 ± 1.78) compared with that in control group (80.25 ± 1.15) ($P<0.01$), whereas the quantity of $OH^{\bullet}$ was decreased in antioxidant NAC group (76.57 ± 1.38) (Table 2). $OH^{\bullet}$ of myoFbs produced by AngII induction was inhibited by NAC.

3.3. *Effect of NAC on content of ROS in myoFbs*

A fluorescence assay was used to detect the expression of ROS. Fig. 1 shows the average fluorescence density of myoFbs measured by computer morphometry. After the treatment with NAC, the values of the mean density decreased. ROS generation of myoFbs induced by AngII was significantly higher than that in control group ($P<0.01$), while the content of ROS in myoFbs in the NAC intervention group was less than AngII group ($P<0.05$). NAC may inhibit the formation of ROS in myoFbs, and thus to some extent to inhibit the proliferation of myoFbs.

3.4. *Translocation and expression of p-PKCα in membrane*

After myoFbs were treated with AngII (10^{-7} M) for 48 h, the expression of *p*-PKCα increased markedly ($P<0.01$ *vs.* control). In Fig. 2A, light yellow represents the nucleus and brown granules indicated *p*-PKCα. Brown particles were more evident in the AngII group, while less apparent in the control group. After treatment with NAC, brown particles clearly decreased compared with those in AngII group ($P<0.05$).

Table 1 Effect of AngII and NAC on the proliferation of myoFbs (mean ± SD).

Group	Proliferation rate of myoFbs (OD value)		
	12 h	24 h	36 h
A	0.295 ± 0.12	0.325 ± 0.15	0.316 ± 0.83
B	0.374 ± 0.13	0.398 ± 0.1[a]	0.387 ± 0.11
C	0.389 ± 0.17	0.483 ± 0.12[a]	0.412 ± 0.14
D	0.341 ± 0.14	0.377 ± 0.13[a]	0.362 ± 0.15
E	0.341 ± 0.14	0.357 ± 0.13	0.339 ± 0.11

[a] $P<0.01$ *vs.* control group.

Table 2 Inhibitory effect of NAC on the content of $OH^{\bullet}$ in myoFbs treated with AngII.

Group	Content of $OH^{\bullet}$ in myoFbs (U/mL)		
	12 h	24 h	36 h
A	78.45±1.23	80.25±1.15	79.80±1.83
C	89.40±1.37[a]	93.53±1.78[a]	90.07±1.16
E	79.31±2.71	76.57±1.38	73.92±1.08

[a] $P<0.01$ *vs.* control group.

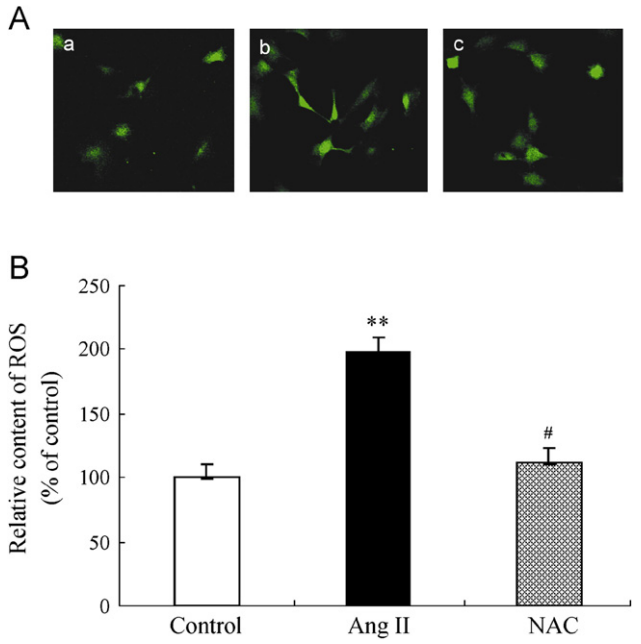

Figure 1 NAC effect on ROS generation of myoFbs with laser confocal scanning microscopy (400×). (a) Control group, (b) AngII (10^{-7} M), and (c) NAC (10^{-4} M). Green density of myoFbs was measured by computer morphometry using the Image-Pro Plus software. Values represent as mean±SD (n=6–8/group). $^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$ *vs.* AngII group.

p-PKCα was found in the membrane and cytoplasm by laser confocal scanning microscopy. In Fig. 3A, the red color stands for nucleus and green particles were *p*-PKCα. In the AngII group, green particles were increased while they were decreased in the control group. After treatment with NAC, green particles decreased compared with those in AngII group. Figs. 2B and 3B show the average density of myoFbs as measured by computer morphometry. After the treatment with NAC, the mean density decreased. NAC could therefore decrease the expression of *p*-PKCα.

3.5. *Phosphorylation of PKCα*

In order to define the mechanisms of how AngII promotes cardiac fibrosis, PKCα phosphorylation was quantified by Western blot analysis (Fig. 4A). It was found that the phosphorylation was significantly increased in the AngII group compared with that in the control group ($P<0.01$).

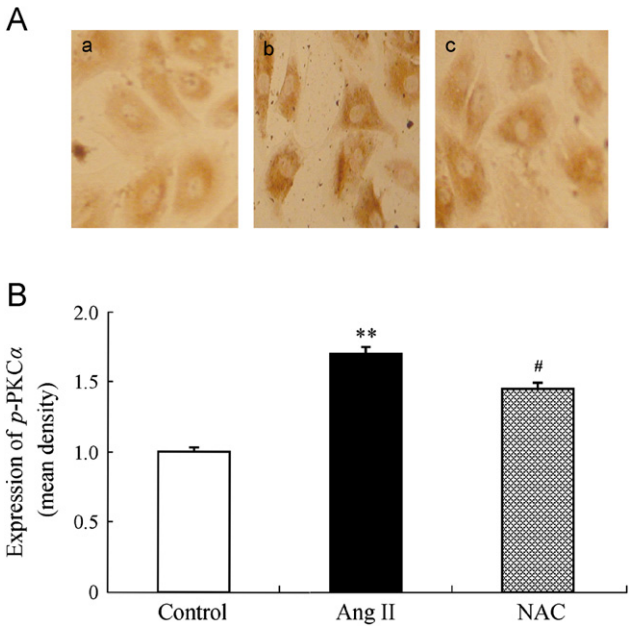

Figure 2 Translocation and expression of *p*-PKCα with immunohistochemistry (200×). (a) Control group, (b) AngII (10^{-7} M), and (c) NAC (10^{-4} M). Brown density of myoFbs was measured by computer morphometry using the Image-Pro Plus software.- Values represent as mean±SD (n=6–8/group). $^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$ *vs.* AngII group.

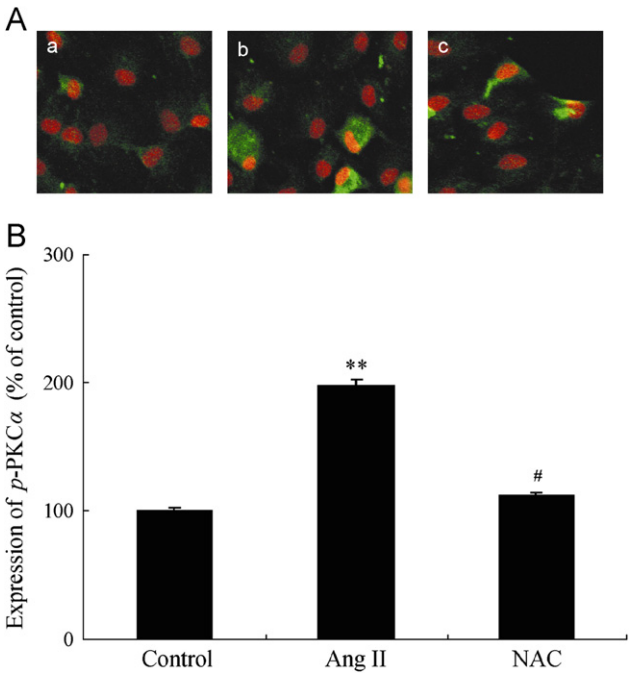

Figure 3 Translocation and expression of *p*-PKCα with laser confocal scanning microscope (400×). (a) Control group, (b) AngII (10^{-7} M), and (c) NAC (10^{-4} M). Brown density of myoFbs was measured by computer morphometry using the Image-Pro Plus software. Values represent as mean±SEM (n=6–8/group). $^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$ *vs.* AngII group.

Phosphorylation of PKCα was reduced in NAC treatment group ($P<0.05$ *vs.* AngII group) (Fig. 4B).

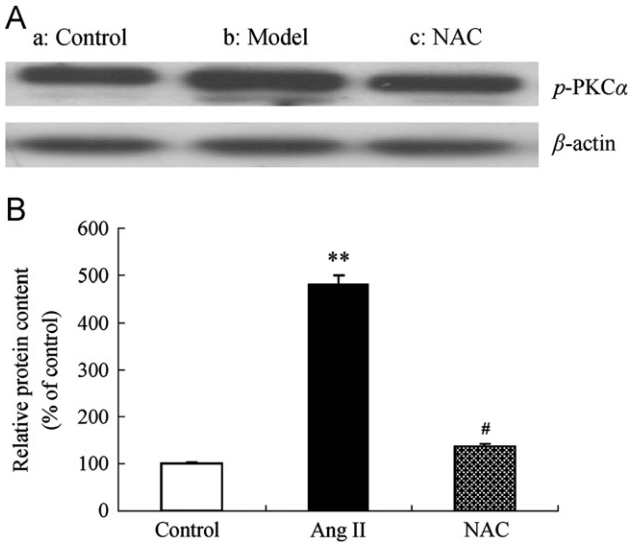

Figure 4 Western blotting analysis of *p*-PKCα in myoFb are shown. β-actin (bottom) was used as control. The lower panels are summary data of *p*-PKCα expression in myoFbs in different groups. (a) Control, (b) AngII (10^{-7} M), and (c) NAC (10^{-4} M). Values were expressed as fold changes over the control and presented as mean ± SD, representative of three independent experiments. $^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$ *vs.* AngII group.

4. Discussion

It is well documented that the abnormal proliferation of myoFbs contributes to the pathogenesis of cardiac fibrosis. On the one hand, myoFbs participate in the process of cardiac fibrosis[10,11], on the other hand, recent studies demonstrate that the inhibition of phenotypic conversion of fibroblasts to myoFbs by cAMP could prevent the cardiac fibrosis[12,13], suggesting a key role of myoFbs in fibrosis. AngII promoted myoFbs proliferation in this study. During pathological conditions of the heart, an activated renin–angiotensin–aldosterone system (RAAS) induces the expression of TGFβ1 that in turn promotes the phenotypic conversion of fibroblasts to myoFbs[14,15]. AngII plays a critical role in the process of cardiac fibrosis[16].

MyoFbs also play a crucial role in the development of cardiac fibrosis due to their over-proliferation. Therefore, inhibiting the proliferation of myoFb is an important anti-fibrotic strategy. Recent research has shown that over-proliferation of myoFb was associated with PKCα[17]. PKCα is expressed ubiquitously among cells and tissues[18–20]. Several lines of evidence indicate that PKCα plays a critical role in cellular functions such as the control of growth, differentiation and apoptosis[21,22]. PKCα was one of the regulatory factors of myocardial fibrosis, and participated in the process of myocardial remodeling[17,23–25]. PKCα is highest in cytoplasm in normal conditions and translocates to the cell membrane when it is phosphorylated in response to numerous factors[24,25]. Many factors mediate the various cellular functions *via* PKC signaling in cardiovascular function and disease[26]. PKC is a group of closely related serine-threonine protein kinases, and PKC isozymes are expressed in all tissues. Members of the PKC family are important signal transducers in virtually every mammalian cell type. In the heart, PKC isozymes were thought to participate in a signal network that programs developmental and pathological myoFbs proliferation. PKC isozymes are involved in a variety of chronic cardiac diseases[27,28] as well as in acute cardiac injuries and preconditioning cytoprotection[25]. Interference with such signaling pathways may result in inhibition of myoFbs proliferation, myocardial fibrosis and reverse cardiac remodeling. To investigate whether AngII induced cardiac fibrosis through PKCα signal transduction pathways, the expression of *p*-PKCα and its translocation was measured with immunohistochemistry and a laser confocal scanning microscopic imaging. The results demonstrate that AngII promoted myocardial fibrosis by activation of PKCα. Under normal conditions, PKCα locates in cytoplasm without activation. PKCα will translocate to membrane when it is activated by AngII to myoFb, and form *p*-PKCα.

A recent study[29–31] showed that reactive oxygen species (ROS) are involved in cell proliferation, differentiation and apoptosis as messenger molecules and participate in the AngII-induced myocardial hypertrophy process. ROS results from aerobic metabolism, generating superoxide anion ($O_2^{\bullet}$), the hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), lipid peroxide intermediate alkoxyl radicals ($LO^{\bullet}$), and alkyl peroxy radicals ($LOO^{\bullet}$). Recent research reported that the hydroxyl radical ($OH^{\bullet}$) could promote ECV304 cell proliferation[29]. ROS are unstable and highly reactive[32] and may serve as a "split signal" second messenger[33], through activation of protein kinase C triggering a series of protein phosphorylation cascade of signal transduction[34] that may be necessary to regulate cell proliferation and ultimately determine the fate of cells[35,36].

NAC is a glutathione precursor which has a strong antioxidant capacity[37]. Treatment with NAC inhibited the proliferation of myoFbs induced by AngII ($P<0.05$). To investigate whether the proliferative effects of AngII are related to ROS, NAC was used to reduce the effects of ROS in this study. From above data, we know that proliferation of myoFbs induced by AngII is inhibited by NAC, suggesting that ROS take part in the AngII-promoted proliferation of myoFbs.

5. Conclusions

AngII could promote the proliferation of myoFbs and resulted in myocardial fibrosis *in vitro*. Our study clearly demonstrates that ROS and *p*-PKCα are involved in the process of myocardial fibrosis induced by AngII. NAC could inhibit the proliferation of myoFbs by decreasing the content of $OH^{\bullet}$ and inhibiting translocation and expression of PKCα. Above all, we found that the proliferation of myoFbs was induced by AngII by way of the ROS–PKCα signal transduction pathways. The current results provide a possible therapeutic target for myocardial fibrosis and to reverse myocardial remodeling induced by AngII through ROS and PKCα signal transduction pathways.

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